Experiments & Protocols

GENERAL PROTOCOLS
DNA PROTOCOLS
PROTEIN PROTOCOLS

***The following methodology is composed by modified methods from last year's team ITESM CEM 2014 (http://2014.igem.org/Team:ITESM-CEM/Project/Materials)**

HEAT-SHOCK TRANSFORMATION OF ASSEMBLED BIOBRICKS

Materials & equipment:

- 1.6 ml microtubes
- Blue and yellow micropipette tips
- Bacteriological handle
- Micropipettes
- Microtubes rack
- Laminar flow hood
- Shaker
- Incubator
- Alcohol lamp

Reagents:

- Competent cells
- Antibiotic (kanamycin 15 mg/ml, chloramphenicol 35 mg/ml or ampicillin 100 mg/ml)
- SOC medium
- Plaques with LB agar
- Liquid LB medium
- Ethanol 96%

Methodology:

1. Add 5 µl of pDNA (concentration between 200-300 pg/ml) to 50 µl competent cells.
2. Incubate 30 min on ice
3. Heat shock 42ºC, 30s
4. Place samples on ice for 5 minutes
5. Add 950 µl SOC medium
6. Incubate at 37ºC for 1 hour, 250 rpm
7. Plate 200 µl of transformed cells into warm, LB agar with the proper antibiotic (kanamycin 15 mg/ml, chloramphenicol 35 mg/ml or ampicillin 100 mg/ml)
8. Incubate overnight at 37ºC (up to 16 h)
9. Isolate a single colony and culture in liquid LB broth for future extractions and plasmid isolation

PLASMID EXTRACTION - MINIPREP

Materials & equipment:

- 15 ml conical tubes
- Microtubes rack
- 100 ml beaker
- Blue and yellow micropipette tips
- 2 ml microtubes
- Micropipettes
- Microcentrifuge
- Shaker
- Boiling water bath

Reagents

- LB liquid media
- Antibiotic (kanamycin 15 mg/ml, chloramphenicol 35 mg/ml or ampicillin 100 mg/ml)
- STET buffer
- Lysozyme (10 mg/ml)
- RNase A (200ug/ml)
- Sodium acetate (3M, pH 5.2)
- Isopropanol
- Ethanol 70%
- Nuclease-free water

Methodology:

1. Culture 2 ml of transformed E.coli into a 50 ml flask containing LB with the proper antibiotic (0.1% v/v).
2. Incubate ON, 37ºC, 250 rpm
3. Place the flask on ice for 20 min
4. Centrifuge the 50 ml of culture at 13000 rpm for 2 minutes
5. Discard supernatant
6. Resuspend biomass in 350 µl of STET buffer
7. Transfer to a 2 ml microtube
8. Add 5 µl of lysozyme (10 mg/ml)
9. Incubate at room temperature for 5 minutes
10. Place the microtube in a boiling water bath during 2 minutes to inactivate the lysozyme.
11. Place sample immediately on ice, centrifuge at 4ºC, 13000 rpm, 10 minutes
12. Take out the bacterial pellet and add 40 µl of RNase A (200ug/ml) to the liquid phase left on the microtube
13. Incubate at room temperature for 10 minutes
14. Add 75 µl of sodium acetate (3M, pH 5.2) and 400 µl of isopropanol
15. Stir gently and incubate for 10 minutes at room temperature
16. Centrifuge at 13500 for 10 minutes and discard the supernatant
17. Wash pellet with 1 ml of ethanol (70%) two times
18. Let pDNA drying for 45 minutes

19. Resuspend in 200 µl of nuclease-free water
20. Quantify by spectrophotometry

VERIFICATION DOUBLE PLASMID DIGESTION PROTOCOL

Materials & equipment:

- Micropipette tips
- Micropipette
- 0.6 ml microtubes
- Thermoblock
- Boiling water bath

Reagents:

- Nuclease-free water
- NEB Buffer 2.1 10X
- Plasmid
- Restriction enzymes

Methodology:

1. Add in a 0.2 ml tube (for a 50 µl reaction):
    a. Control 1:
        i. Up to 50 µl nuclease-free water
        ii. 5 µl Buffer 10X
        iii. 500 µg Plasmid
        iv. 1 µl Restriction enzyme 1
    b. Control 2:
        . Up to 50 µl nuclease-free water
        i. 5 µl Buffer 10X
        ii. 500 µg Plasmid with Promoter
        iii. 1 µl Restriction Enzyme 2
    c. Digestion:
        . Up to 50 µl nuclease-free water
        i. 5 µl Buffer 10X
        ii. 500 µg Plasmid with Promoter
        iii. 1 µl Restriction Enzyme 1
        iv. 1 µl Restriction Enzyme 2
2. Mix gently each tube
3. Place at thermoblock, 37ºC for 1 hour
4. Inactivate enzymes at 80ºC for 20 minutes
5. Store digestion products at -20ºC

LIGATION

Materials & equipment:

- Micropipette tips
- Micropipette
- 0.6 ml microtubes
- Boiling water bath

Reagents:

- Nuclease-free water
- DNA
- 10X T4 DNA Ligase Buffer
- T4 DNA Ligase

Methodology:

1. For a 20 µl reaction. Add in the following order:
    a. 11 µl nuclease-free water
    b. 3 µl Digestion 1
    c. 3 µl Digestion 2
    d. 2 µl 10X T4 DNA Ligase Buffer
    e. 1 µl T4 DNA Ligase
2. Incubate at room temperature (20ºC - 25ºC) for 2 hours
3. Heat inactivate at 80ºC for 20 minutes
4. Transform 10 µl ligation into 50 µl of competent cells
5. Store the rest at -20ºC

LIGATION INTERLAB 2015

Materials & equipment:

- Micropipette tips
- Micropipette
- 0.6 ml microtubes
- Boiling water bath

Reagents:

- Nuclease-free water
- DNA
- 10X T4 DNA Ligase Buffer
- T4 DNA Ligase

Methodology:

1. For a 20 µl reaction. Add in the following order:
    a. 11 µl nuclease-free water
    b. 4 µl Promoter digestion
    c. 2 µl BBa_I13504 digestion
    d. 2 µl 10X T4 DNA Ligase Buffer
    e. 1 µl T4 DNA Ligase
2. Incubate at room temperature (20ºC - 25ºC) for 2 hours
3. Heat inactivate at 80ºC for 20 minutes
4. Transform 10 µl ligation into 50 µl of competent cells
5. Store the rest at -20ºC

AGAROSE GEL ELECTROPHORESIS

Materials & equipment:

- 250 ml conical flask
- Micropipette tips
- Micropipette
- Microwave
- Casting trays and gel comb
- Agarose gel box and power source
- Imager

Reagents:

- 1X TAE Buffer
- Agarose
- GelRed
- Loading Buffer

Methodology:

1. Add 50 ml 1X TAE to a conical flask.
2. Measure out sufficient agarose to cast either a 0,7% (0,35 g), 1,0% (0,50 g) or 1,2% (0,60 g) gel.
3. Add the agarose to the TAE buffer in the conical flask.
4. Swirl to mix.
5. Microwave the flask on high until the gel starts to bubble and is transparent.
6. Let cool by either sitting on bench top.
7. While gel is cooling, assemble casting trays and gel combs and verify that the trays are level.
8. Once gel is cooled so that it can be touched comfortably with your gloved hand, add 3ul of GelRed (NOTE: GelRed is an acute toxin and a strong mutagen, be cautious and dispose gloves immediately after handling it!)
9. Pour gel into casting trays.
10. Let gel sit until they are solidified.
11. Remove comb.
12. Place your gel in gel box.
13. Add 1X TAE buffer to gel box such that buffer just covers the top of the gel.
14. Load 10 µL of prepared ladder.
15. Mix 2 µL of loading dye with 10 µL of sample.
16. Place gel box cover on gel box such that your samples will run towards the positive, red electrode.
17. Run your gel at ~70□80 volts for 45□60 min (NOTE: running voltage and time depend on the agarose percentage and length of the DNA samples).
18. Visualize the gel with a gel imager.

RECOMBINANT PROTEIN EXPRESSION

Materials & equipment:

- 1.5 ml sterile microtubes
- 15 ml conical tubes
- 250 ml sterile conical flask
- Micropipette tips
- Micropipette
- Laminar flow hood
- Shaker
- Centrifuge

Reagents:

- LB liquid medium
- IPTG 1 mM

Methodology:

1. Inoculate a 5 ml tube with LB broth and the proper antibiotic. Incubate at 37ºC overnight.
2. Inoculate those 5 ml into a 50 ml flask with LB broth and the proper antibiotic. Incubate 1 hour at 37ºC, 200 rpm.
3. Take 1 ml sample, place on ice.
4. Add 1mM IPTG (50 µl) and take another 1 ml sample
5. Return to incubating conditions and take a sample each hour for 6 hours
6. Centrifuge all samples and recover biomass
7. Resuspend the biomass in Laemmli buffer, then put the samples into boiling water for 5 minutes
8. Cool the samples at room temperature and then put them on ice
9. SDS PAGE analysis is performed

RECOMBINANT PROTEIN SOLUBILITY ANALYSIS

Materials & equipment:

- 15 ml conical tubes
- 1.5 ml microtubes
- Micropipettes

Reagents:

- 20 mM K2HPO4 and 10 mM KCl (pH 8) solution
- 300 mM KCl, 20 mM Imidazole (pH 8) and 20 mM KH2PO4 solution
- Laemmli Buffer
- Lysozyme (10 mg/ml)

Methodology:

1. Centrifuge 40 mL medium 5000 rpm, 30 min. Remove supernatant.
2. Resuspend pellet in 4 mL of a cold 300 mM KCl (pH 8), 20 mM Imidazole (pH 8) and 20 mM KH2PO4 solution.
3. Add 70uL of Lysozyme.
4. Incubate in shaker 200 rpm the biomass-lysozyme solution at 37° C for 60 minutes.
5. Centrifuge cell lysate at 14,000 rpm for 10 minutes. If the supernatant is not crystalline, the sample has to be centrifuged again. Store soluble phase at 4°C in a conical tube, covered with aluminum for chromatography.
6. Take 20 µL of the supernatant and mix with 20 µL of Laemmli buffer for SDS PAGE analysis.
7. The insoluble phase obtained from the previous centrifugation has to be washed twice with a 1% SDS solution and 20 mM KH2PO4 and 10 mM KCl (pH 8) solution. Then, mix insoluble phase with 20ul Laemmli buffer for SDS PAGE analysis.
8. Run a SDS PAGE with iGEM ITESM CEM's protocol.

*Do not cease to contemplate that all samples should be perfectly mixed with the indicated reagents (Laemmli Buffer) before they are boiled for 5 minutes. In the case of the inclusion bodies, the boiling should be carried out for 10 minutes.

SDS PAGE 15%

Materials & equipment:

- 2 microtubes
- 1 micropipettes set
- 2 beakers (50 ml)
- SDS-PAGE glasses (with spacers 1.0 mm), comb and module assembly
- Mini-Protean System

Reagents:

- Resolving Buffer (1.5M Tris-HCl, pH 8.8)
- Stacking Buffer (0.5 M Tris-HCl pH 6.8)
- Running buffer 1X (Tris-Glycine/SDS)
- Laemmli buffer
- Blue Coomasie Solution
- Destaining solution
- Deionized water
- SDS 10%
- APS 10%
- TEMED
- Molecular weight protein marker (Precision Plus Protein™ All Blue Prestained Protein Standards)

Methodology:

***Use gloves at ALL times. Acrylamide and Bis-acrylamide are extremely toxic.**

1. Place glasses on the module assembly
2. Prepare resolving gel solution (12%) as follows by adding the reagents in the following order
    - 1.875 ml acrylamide/bis-acrylamide 30%
    - 1.25 ml Resolving buffer
    - 0.05 ml SDS 10%
    - 1.12 ml Deionized water
    - 65 µl APS 10%
    - 15 µl TEMED

      *** The TEMED has to be added until the end, and immediately proceed to the next step, because polymerization will be initiated.**
3. Slowly add the resolving gel solution between the panes (about 5 ml)
4. Carefully add deionized water. This avoids the presence of oxygen, otherwise polymerization can be inhibited
5. Wait 15-20 minutes until the gel is solid. Dry the water added with filter paper
6. Prepare stacking gel solution (5%) as follows by adding the reagents in the following order
    - 0.375 ml acrylamide/bis-acrylamide 40%
    - 1.25 ml Stacking buffer
    - 0.05 ml SDS 10%

- 3 ml Deionized water
- 30 µl APS 10%
- 15 µl TEMED

  *** The TEMED has to be added until the end, and immediately proceed to the next step, because polymerization will be initiated.**

7. Slowly add the resolving gel solution between the panes (about 1.5 ml)
8. Install the comb, taking care not to trap any oxygen.
9. Wait 15-20 minutes until the gel is solid.
10. Place the gel in the electrophoresis chamber, ensure that the smaller glass is facing inwards
11. Fill the chamber with running buffer 1X until it covers the gel and the lower chamber until the appropriate mark
12. Load 10 µl of protein marker. Then, load 20 ul of protein sample previously mixed with 20 ul Laemmli Buffer.
13. Run gel. 15 minutes at 100 V and then increase to 150 V for 60 minutes
14. Turn off power source and remove gel from camera
15. Stain gel with Coomasie Blue Solution overnight. Cover with aluminium.
16. Remove Coomasie Blue Solution and stirr the gel in destaining solution for 30 minutes. Wash again for 30 minutes with destaining solution.
17. Visualize protein.

PURIFICATION OF A RECOMBINANT PROTEIN

Materials & equipment:

- 1.6 ml microtubes
- 15 ml conical tubes
- Micropipettes
- Spectrophotometer cells
- Bombing system
- Fraction collector
- Chromatography column with Ni affinity
- Spectrophotometer
- 15% SDS gel
- Electrophoresis chamber

Reagents:

- Soluble fraction of a 500 ml culture
- Interaction buffer: NaH2PO420 mM and NaCl 10 mM, pH 8.0 (Buffer A)
- Elution buffer: NaH2PO4 20 mM and NaCl 300 mM, Imidazol 20 mM pH 8.0 (Buffer B)

Methodology:

1. Resuspend the fraction of interest in interaction buffer
2. Previously prepare the chromatography column with 2.5 ml of Ni resin Agarose 6 Fast Flow. After that, the column has to be washed for 10 minutes with deionized water and then equilibrate the system washing the column with 10 ml of the interaction buffer.
3. Connect the column to the to the chromatograph and inject the sample with a flow of 0.5 ml/min.
4. Program the equipment. First wash the column with 7.5 ml of the same interaction buffer, then a gradient from 20% to 80% of both buffers A and B; at the end 2.5 ml of 100 % buffer B. The column has to be re-equilibrated with 5 ml of buffer A. The flow rate of the whole process will be 1 ml/min and 1 ml of each fraction will be collected.
5. All the fractions have to be read in a spectrophotometer at 280 nm to identify the chromatographic peak of elution, that corresponds to the purified protein.
6. All the samples that match the peak of elution, will be prepared to be run in a SDS-PAGE gel.